

## Concentricolide, an Anti-HIV Agent from the Ascomycete *Daldinia concentrica*

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A novel benzofuran lactone, named concentricolide (= *rel*-(6*R*)-6-ethylbenzo[2,1-*b*:3,4-*c'*]difuran-8(6*H*)-one; **1**), was isolated along with four known compounds (friedelin, cytochalasin L-696,474, armilaramide, and russulamide) from the fruiting bodies of the xylariaceous ascomycete *Daldinia concentrica*. The structure of **1** was established by spectroscopic methods and X-ray crystallographic analysis. Its anti-HIV-1 activity was tested. Results showed that **1** inhibited HIV-1 induced cytopathic effects. The  $EC_{50}$  value was 0.31  $\mu\text{g/ml}$ . The therapeutic index (*TI*) was 247. Concentricolide exhibited the blockage ( $EC_{50}$  0.83  $\mu\text{g/ml}$ ) on syncytium formation between HIV-1 infected cells and normal cells.

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**Introduction.** – Although anti-HIV-1 drugs now available have improved the quality of the lives of HIV/AIDS patients, the rapid evolution of new HIV clades and drug resistant variants in AIDS patients urged the search for new anti-HIV-1 agents and targets. A large variety of natural products including alkaloids, flavonoids, coumarines, lignans, phenolics, triterpenoids, saponins, sulfated polysaccharides, phospholipids, quinines, and peptides with anti-HIV-1 effect have been described, and for a portion thereof the target of interaction has been identified [1–4]. Natural products provide a large reservoir for screening of anti-HIV-1 agents with novel structure and antiviral mechanisms.

Yunnan Province, southwest of China, is one of the areas with the richest and diverse bio-resources in the world. Fungi in these bio-resources belong to the most productive biological sources producing a large and diverse variety of secondary metabolites. As one part of our search for bioactive metabolites of the higher fungi in Yunnan Province [5] [6], the chemical constituents of Chinese *Daldinia concentrica* collected at Lijing of Yunnan were investigated. *D. concentrica* has afforded a great deal of scientific interest because of its unique secondary metabolites. *Allport* and *Bu'Lock* have studied European and American *Daldinia* sp. in 1958 and 1960 [7] [8], which had resulted in the identification of characteristic metabolites in their stromata and cultures. Some of these compounds were found to have antimicrobial and nematocidal

activities [9]. During more recent studies on *Daldinia* sp., more than 20 new metabolites have been discovered, including aromatic steroids [10], azaphilone [11], benzophone [12], benzoquinones [13], a binaphthyl [12], cytochalasins [14–16], a daldiniapyrone [17], daldnones [17], heptentriol [18], and triterpenoids [17][19][20], of which some show a range of biological activities.

We now isolated one novel benzofuran derivative, named concentricolide (**1**), together with four known compounds, from the fungus *D. concentrica*. This report deals with the characterization and structure elucidation of these compounds and the anti-HIV-1 activity of concentricolide (**1**). The structure of **1** (Fig. 1) was established by spectroscopic methods and X-ray crystallographic analysis. Results showed that **1** inhibited HIV-1-induced cytopathic effects. The  $EC_{50}$  value was 0.31  $\mu\text{g/ml}$ . The therapeutic index ( $TI$ ) was  $> 200$ . Concentricolide (**1**) exhibited the blockage ( $EC_{50}$  0.83  $\mu\text{g/ml}$ ) on syncytium formation between HIV-1-infected cells and normal cells. The results from this study suggested that **1** is effective against HIV-1 [21].

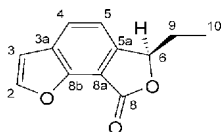


Fig. 1. Structure of concentricolide (**1**)

**Results and Discussion.** – Compound **1** was obtained as pale yellow needles. The IR spectrum showed absorptions at 1757 ( $\gamma$ -lactone), 1641, 1534, and 1437 (aromatic ring)  $\text{cm}^{-1}$ . The molecular formula was established as  $\text{C}_{12}\text{H}_{10}\text{O}_3$  on the basis of the mass spectrum and DEPT  $^{13}\text{C}$ -NMR spectroscopic data (Table) and confirmed by high resolution TOF-MS, which displayed a peak at  $m/z$  225.0526 ( $[M + \text{Na}]^+$ ). Further data suggested the structure of a benzofuran fused to an ethyl-substituted  $\gamma$ -lactone moiety. This was confirmed by the X-ray crystallographic analysis of **1** which also established the relative configuration at C(6) (Fig. 2).

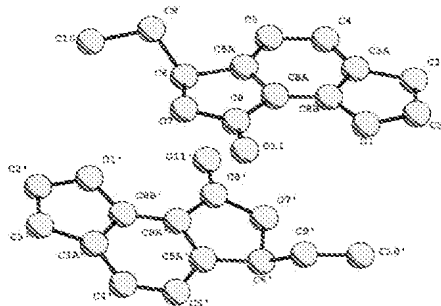


Fig. 2. X-Ray stereo drawing of concentricolide (**1**)

Twelve signals in the  $^{13}\text{C}$ -NMR (DEPT) spectra of **1** were recognized (5C, 5CH, 1CH<sub>2</sub>, 1Me), including one ester carbonyl C-atom ( $\delta$  168.1), four aromatic quaternary C-atoms ( $\delta$  110.9, 128.9, 147.8, 149.6), and four aromatic CH groups ( $\delta$  106.6, 116.0, 127.8, 146.4). The remaining signals in the  $^{13}\text{C}$ -NMR spectra of **1** could be attributed to 1 Me ( $\delta(\text{H})$ , 0.95 ( $t$ ,  $J = 7.2$  Hz);  $\delta(\text{C})$ , 8.7), 1 CH<sub>2</sub> ( $\delta(\text{H})$ , 1.81, 2.13 (each  $m$ , 1

Table.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR Data ( $\text{CD}_3\text{OD}$ ) of Compound 1.  $\delta$  in ppm,  $J$  in Hz.

	$\delta(\text{C})$	$\delta(\text{H})$	$^1\text{H}, ^1\text{H}$ -COSY	HMBC (selected)
H–C(2)	146.4	7.74 ( $d, J=2.2$ )	H–C(3)	H–C(3)
H–C(3)	106.6	6.86 ( $d, J=2.2$ )	H–C(2)	H–C(2), H–C(4)
C(3a)	128.9			H–C(2), H–C(5)
H–C(4)	127.8	7.86 ( $d, J=8.3$ )	H–C(5)	H–C(3)
H–C(5)	116.0		H–C(4)	
C(5a)	147.8			H–C(4), $\text{MeCH}_2$
H–C(6)	82.9	5.52 ( $dd, J=7.1, 4.1$ )	$\text{MeCH}_2$	H–C(5), $\text{MeCH}_2$ , $\text{MeCH}_2$
C(8)	168.1			H–C(6)
C(8a)	110.9			H–C(5), H–C(6)
C(8b)	149.6			H–C(2), H–C(3), H–C(4)
$\text{MeCH}_2$	27.8	1.80–1.82 ( $m, 1\text{ H}$ ), 2.12–2.14 ( $m, 1\text{ H}$ )	H–C(6), $\text{MeCH}_2$	H–C(6), $\text{MeCH}_2$
$\text{MeCH}_2$	8.7	0.95 ( $t, J=7.2$ )	$\text{MeCH}_2$	H–C(6), $\text{MeCH}_2$

H);  $\delta(\text{C})$ , 27.8), and 1 OCH group ( $\delta(\text{H})$ , 5.52 ( $dd, J=7.1, 4.1\text{ Hz}$ );  $\delta(\text{C})$ , 82.9 (C(6)). The  $^1\text{H}, ^1\text{H}$ -COSY data (Table) allowed establishment of three H-atom systems, one at C(2) through C(3), one at C(4) through C(5), and one at C(6) through  $\text{MeCH}_2$ , by showing the correlations H–C(2)/H–C(3); H–C(4)/H–C(5); H–C(6)/ $\text{MeCH}_2$ , and  $\text{MeCH}_2/\text{MeCH}_2$ . This was confirmed by the HMBC correlations (Table) C(3a)/H–C(2) and H–C(5), C(8b)/H–C(2), H–C(3), H–C(4), and C(8a)/H–C(5), and C(8)/H–C(6).

Comparison of the physicochemical properties with reported data allowed to identify four other compounds isolated from the same fungus as friedelin [22], L-696,474 [23], armillaramide [24], and russulamide [25].

The cytotoxicity of concentricolide (1) is shown in Fig. 3. The  $\text{CC}_{50}$  of 1 was 76.66  $\mu\text{g/ml}$  (379.50  $\mu\text{M}$ ) and  $\text{EC}_{50}$  of 1 on inhibiting the HIV-1-induced cytopathic effect (Fig. 4) was 0.31  $\mu\text{g/ml}$  (1.53  $\mu\text{M}$ ). The  $\text{TI}$  of concentricolide was *ca.* 247. The blockage

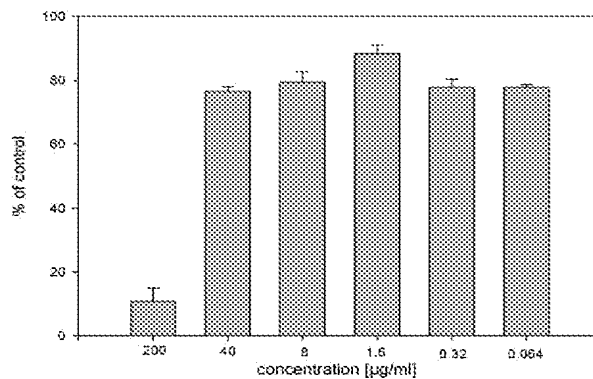


Fig. 3. Cytotoxicity of concentricolide (1) on C8166 cells ( $\text{CC}_{50}=76.66\text{ }\mu\text{g/ml}$ ) with respect to that of azidothymidine (AZT) as positive control drug ( $\text{CC}_{50}=258.88\text{ }\mu\text{g/ml}$ )

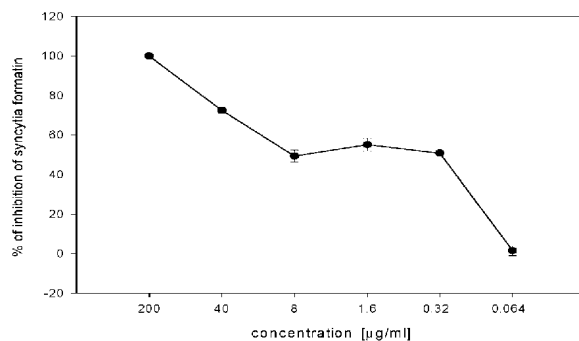


Fig. 4. *Cytopathic-effect (CPE) inhibition by concentricolide (1)* ( $EC_{50}=0.31\ \mu\text{g/ml}$ ). CPE inhibition by AZT (positive control drug):  $EC_{50}=0.092\ \mu\text{g/ml}$ .

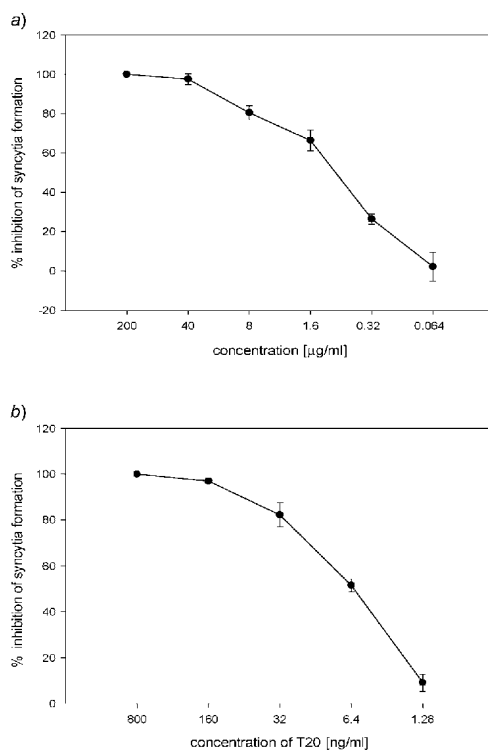


Fig. 5. a) *Blockage of concentricolide (1) on syncytium formation between HIV-1 infected cells and normal cells* ( $EC_{50}=0.83\ \mu\text{g/ml}$ ). b) *Blockage of T20 (positive control drug) on syncytium formation between HIV-1-infected cells and normal cells* ( $EC_{50}=6.02\ \text{ng/ml}$ ).

of **1** on syncytium formation between HIV-1 infected cells and normal cells was also measured (Fig. 5), the  $EC_{50}$  being 0.83  $\mu\text{g/ml}$  (4.10  $\mu\text{M}$ ).

In summary, a new derivative of benzofuran lactone, named concentricolide (**1**), was isolated from the fruiting bodies of the ascomycete *Daldinia concentrica*. The structure and relative configuration of the new compound was elucidated by detailed spectroscopic analysis and confirmed by X-ray crystallography. Our data demonstrated that **1** is a compound with *in vitro* activity against HIV-1. The mechanism of anti-HIV activity of **1** needs to be further investigated, and this study will be focused on the entry step of HIV-1.

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### Experimental Part

**General.** CC=Column chromatography. M.p.: *XRC-1* apparatus (Sichuan University, Sichuan, China). Optical rotations: *Horiba-Sepa-300* automatic polarimeter (*Horiba*, Tokyo, Japan). UV Spectra:  $\lambda_{\text{max}}$  in nm. IR Spectra: KBr pellets; *Bio-Rad-FTS-135-IR* spectrophotometer (*Bio-Rad*, Richmond, CA);  $\tilde{\nu}$  in  $\text{cm}^{-1}$ . NMR Spectra ( $^1\text{H}$ ,  $^{13}\text{C}$ , and 2D): *DRX-500* instrument (*Bruker*, Karlsruhe, Germany); at 500 ( $^1\text{H}$ ) and 125 MHz ( $^{13}\text{C}$ );  $\delta$  in ppm rel. to  $\text{SiMe}_4$  as internal standard, coupling constants  $J$  in Hz. Mass spectra: *VG-Autospec-3000* mass spectrometer (*VG*, Manchester, England) and *API Qstar Pulsar* (*Applied Biosystems*, Foster City, USA); in  $m/z$  (rel. %). X-ray crystallographic analysis was performed with a *MAC-DIP-2030K* diffractometer.

**Reagents, Cells, and Virus.** AZT (=3'-azido-3'-deoxythymidine) and MTT (=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from *Sigma*. SDS (=sodium dodecyl sulfate) was from *Serva* and DMF (dimethylformamide) from *Shanghai Chemical Reagents Company* (China). Complete RPMI-1640 medium was supplemented with 10% heat-inactivated fetal calf serum (*Gibco*), 2 mM L-glutamine, 10 mM HEPES (=4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 50  $\mu\text{M}$  2-mercaptoethanol, 100 000 IU/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin sulfate. AZT, a reverse-transcriptase inhibitor, and T20 (enfuvirtide), an entry inhibitor were used as positive control in anti-HIV-1-activity experiments. C8166 and HIV-1<sub>IIIB</sub> chronically infected H9 cells were donated by the Medical Research Council (MRC), AIDS Reagent Project, UK. All cells and virus were stored and resuscitated by common methods.

**Cytotoxicity of Concentricolide (**1**) on C8166 Cells.** C8166 was one of the host cells for HIV-1. On a microtiter plate, 100  $\mu\text{l}$  of  $4 \cdot 10^5/\text{ml}$  cells were seeded. Then 100  $\mu\text{l}$  of various concentrations of **1** were added and incubated at 37° in a humidified atmosphere of 5%  $\text{CO}_2$  for 72 h. The cellular toxicity was assessed by a MTT colorimetric assay, the plates were read with a *Bio-Tek ELx 800* ELISA reader at 595/630 nm, and the 50% cytotoxic concentration ( $CC_{50}$ ) was calculated.

**Cytopathic-Effect (CPE) Inhibition Assay of Concentricolide (**1**).** In the presence of 100  $\mu\text{l}$  of various concentrations of **1**, C8166 cells ( $4 \cdot 10^5/\text{ml}$ ) were infected with HIV-1<sub>IIIB</sub> at a multiplicity of infection (M.O.I.) of 0.06. The final volume was 200  $\mu\text{l}$ . The plates were incubated in a humidified incubator at 37° and 5%  $\text{CO}_2$ . AZT was used for drug control. After 3 d of culture, the cytopathic effect was measured by counting the number of syncytia (multinucleated giant cell) in each well under an inverted microscope, and the 50% effective concentration ( $EC_{50}$ ) was calculated.

**Blockage of Concentricolide (**1**) on Syncytium Formation between HIV-1-Infected Cells and Normal Cells.** When HIV-infected cells were co-cultured with normal T lymphocyte cells, the exterior envelope glycoprotein gp120 expressed on infected cells bind to the cellular CD4 receptor of the uninfected  $\text{CD4}^+$  cells, following fusion of cells and formation of syncytia. Compounds target to this site would inhibit the syncytia formation. Thus, this method could be used to detect whether the compounds have effect on the

entry of virus. Briefly, 100  $\mu$ l of serial five-fold concentricolide-containing medium was added to a 96-well microtiter plate. Then, 50  $\mu$ l of C8166 cells ( $6 \cdot 10^5$ /ml) and 50  $\mu$ l of HIV-1<sub>IIIB</sub> chronically infected H9 cells ( $2 \cdot 10^5$ /ml) were added. The plate was cultured in a humidified incubator at 37° and 5% CO<sub>2</sub> for 24 h, and the syncytial formation was quantified under an inverted microscope. T20 (enfuvirtide) was used as a positive control.

**Fungal Materials.** Fruiting bodies of *Daldinia concentrica* were collected at Lijiang, Yunnan Province, P. R. China, in 2003. The voucher specimen was deposited at the herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences.

**Extraction and Isolation.** Dried fruiting bodies (750 g) of *Daldinia concentrica* were extracted 3 $\times$  with CHCl<sub>3</sub>/MeOH 1:1 (v/v) and 3 $\times$  with MeOH at r.t. The combined org. phase was evaporated to a small volume to afford a deep brown gum (77 g), which was submitted to CC (silica gel, CHCl<sub>3</sub>/MeOH 100:0, 95:5 (v/v)). **Fractions 1–12.** Friedelin (6.3 mg) was obtained from *Fr.* 5 (CHCl<sub>3</sub>/MeOH 95:5) and russulamide (13.5 mg) from *Fr.* 12 (CHCl<sub>3</sub>/MeOH 95:5). *Fr.* 8 (CHCl<sub>3</sub>/MeOH 95:5; 9 g) was resubjected to CC (silica gel, petroleum ether/acetone 99:1, 95:5, 90:10, and 80:20): **1** (120 mg; with petroleum ether/acetone 99:1), and L-696,474 (240 mg) and armillaramide (5 mg), both with petroleum ether/acetone 80:20.

**Data of Concentricolide** (=rel-(6R)-6-Ethylbenzo[2,1-b:3,4-c']difuran-8(6H)-one; **1**): Pale yellow needles. M.p. 89–90° (petroleum ether/acetone).  $[\alpha]_D^{25} = -59.2$  ( $c = 0.48$ , MeOH). UV(MeOH): 226. IR: 1757, 1641, 1534, 1437. <sup>1</sup>H- and <sup>13</sup>C-NMR: *Table*. EI-MS: 202 (20, *M*<sup>+</sup>), 173 (100), 145 (48). HR-TOF-MS: 225.0526 ( $[M + Na]^+$ , C<sub>12</sub>H<sub>10</sub>NaO<sub>3</sub>; calc. 225.0527).

**X-Ray Crystallographic Study.** Crystal data: C<sub>12</sub>H<sub>10</sub>O<sub>3</sub>, *M*<sub>r</sub> 202.2, triclinic, space group *P*1;  $a = 7.728(1)$ ,  $b = 8.289(1)$ ,  $c = 9.043(1)$  Å;  $\alpha = 106.450(5)^\circ$ ,  $\beta = 96.321(6)^\circ$ ,  $\gamma = 108.946(6)^\circ$ ;  $V = 512.26(11)$  Å<sup>3</sup>,  $Z = 2$ . Final *R* and *wR* values were 0.081 and 0.193, resp. A total of 1712 reflections were recorded in the  $\omega$  scanning mode with a MAC-DIP-2030K diffractometer with graphite-monochromated MoK $\alpha$  scanning radiation. The structure was solved by the direct methods (SHELXS-97).

CCDC-211009 contains supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via [http://www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif).

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